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Mast cell-derived mediators promote murine neutrophil effector functions

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Abstract

Mast cells are able to trigger life-saving immune responses in murine models for acute inflammation. In such settings, several lines of evidence indicate that the rapid and protective recruitment of neutrophils initiated by the release of mast cell-derived pro-inflammatory mediators is a key element of innate immunity. Herein, we investigate the impact of mast cells on critical parameters of neutrophil effector function. In the presence of activated murine bone marrow-derived mast cells, neutrophils freshly isolated from bone marrow rapidly lose expression of CD62L and up-regulate CD11b, the latter being partly driven by mast cell-derived TNF and GM-CSF. Mast cells also strongly enhance neutrophil phagocytosis and generation of reactive oxygen species. All these phenomena partly depend on mast cell-derived TNF and to a greater extent on GM-CSF. Furthermore, spontaneous apoptosis of neutrophils is greatly diminished due to the ability of mast cells to deliver antiapoptotic GM-CSF. Finally, we show in a murine model for acute lung inflammation that neutrophil phagocytosis is impaired in mast cell-deficient *Kit^{W-sh}/Kit^{W-sh}* mice but can be restored upon mast cell engraftment. Thus, a previously underrated feature of mast cells is their ability to boost neutrophil effector functions in immune responses.

Keywords: cell activation, inflammation, lung, mast cells, neutrophils, rodent

Introduction

Polymorphonuclear neutrophils are the most abundant leukocyte population (50–70%) in the peripheral blood of humans and make important contributions to the innate host defense against pathogenic micro-organisms such as fungi and bacteria. They participate in the early innate immune response by rapidly migrating into inflamed tissue, where they employ their potent effector functions such as phagocytosis, the release of anti-microbial substances, reactive oxygen species (ROS) and inflammatory mediators. The effector functions of neutrophils are mainly mediated and regulated via cell-surface receptors (i.e. fMLP receptors and TLRs) (1–3). However, under inflammatory conditions and also in homeostasis, it is important that the activation and survival of neutrophils is tightly controlled since the release of cytotoxic substances by neutrophils

can easily cause collateral damage of adjacent healthy tissue (4).

Apoptosis is an important mechanism of safely eliminating neutrophils during the resolution of inflammation. In parallel with the activation of neutrophil effector functions, many inflammatory mediators also regulate cell survival by altering apoptosis (5, 6). The local control of neutrophil activation and survival is highly relevant for all inflammatory conditions, from microbial infections to sterile inflammations such as gout (7) or rheumatoid arthritis where neutrophils make a significant contribution. Therefore, it is important to understand the underlying mechanisms to control local neutrophil activity.

With the advent of mast cell-deficient mice and selective engraftment with *in-vitro*-generated bone marrow-derived mast cells (BMMC), it was shown that these cells are critical

for the initiation of acute inflammatory responses in diverse experimental settings. In several models for acute inflammation in mice, the rapid recruitment of neutrophils turned out to be initiated by mast cells, which are predominantly localized at possible entry sites of noxious substances (8–11).

With regard to the underlying mechanisms, the influx of neutrophils in immune-complex-mediated peritonitis in mice was reported to partly depend on mast cell-derived leukotrienes (LT) as potent chemoattractants (12).

In addition, mast cell-derived TNF was shown to be crucial for the recruitment of neutrophils in this model and also in late-phase cutaneous anaphylaxis (13, 14), contact hypersensitivity reactions (CHSR) (15), T_H17 cell-dependent lung inflammation (16), formation of cutaneous granulomas (17), experimental autoimmune encephalomyelitis (18) and IL-33-induced inflammation (19). The unique ability of mast cells to store and immediately release TNF on demand is essential for the rapid onset of inflammatory reactions (20, 21).

A cornerstone in this context was the observation that mast cells and mast cell-derived TNF initiate the life-saving influx of neutrophils in mouse models for acute bacterial infections (22, 23). In murine infectious peritonitis, it was published that besides TNF, mast cell-derived LT, mouse mast cell protease 6 and the chemokine macrophage inflammatory protein 2 (MIP-2) are critical for a rapid and protective influx of neutrophils (24–27). In a murine model for CHSR, it was shown that mast cell-derived TNF acts on TNFRI-expressing endothelial cells mediating the recruitment of neutrophils to sites of inflammation (28). In addition, mast cell-derived MIP-2 acts synergistically with TNF by establishing a chemotactic gradient for neutrophil extravasation and migration (15). The ability of mast cells to participate in innate immunity is based on the expression of a host of receptors leading to the activation of these cells independently of IgE, including most TLRs and complement receptors (29, 30).

Herein, we investigate the impact of murine BMMC and mast cell-derived TNF and GM-CSF on critical effector functions of freshly isolated neutrophils. Activated BMMC modulate the expression of activation markers on neutrophils, trigger generation of ROS, promote phagocytosis and reduce spontaneous apoptosis. Importantly, in mast cell-deficient mice, neutrophil phagocytosis is impaired in a model for acute lung inflammation.

Methods

Mice

Genetically mast cell-deficient *Kit^{W-sh}/Kit^{W-sh}* mice on a C57BL/6 background (31–33) were initially obtained by Marcus Maurer (Department of Dermatology, Charité, Berlin, Germany). Mice deficient for TNF (34) were kindly provided by Kerstin Steinbrink (Department of Dermatology, Mainz, Germany). TNF-deficient animals were adequately backcrossed to the C57BL/6 strain and have been used as bone marrow and mast cell donors in several of our prior publications (35, 36). Mice deficient for GM-CSF (37) on a C57BL/6 background were provided by L.C. (Institute of Experimental Immunology, Zürich, Switzerland). Animal procedures were conducted in accordance with the institutional guidelines.

Generation and activation of BMMC and reconstitution of mast cell-deficient mice

BMMC were generated from bone marrow according to standard procedures (38). For stimulation via their FcεRI receptors, BMMC were sensitized with the IgE anti-DNP Ab A2 for 48–72 h (39), washed and then cross-linked using plate-bound 2,4-dinitrophenyl human serum albumin (DNP-HSA) (2.5 μg ml⁻¹; Sigma-Aldrich, Seelze, Germany) for 24 h. *Kit^{W-sh}/Kit^{W-sh}* mice were systemically given intravenous injections of 5 × 10⁶ BMMC for at least 12 weeks before the experiments were conducted. To assess reconstitution efficiencies, lungs were fixed by inflation (1 ml), immersed in 4% formalin or Carnoy's solution and embedded in paraffin. Tissue sections were used for metachromatic mast cell stainings with toluidine blue (40). Slides were examined in a blinded fashion with a microscope (BX40; Olympus, Hamburg, Germany). For the assessment for mast cell numbers in each slide, mast cells were counted by a blinded investigator in five different fields and in each field the lung area was measured using an image analysis system (Soft Imaging System; Olympus). Numbers of mast cells are expressed per square centimeter.

Flow cytometric analyses

Flow cytometry studies were performed on FACSCanto and LSR II analyzer running FACSDiva software (BD, Heidelberg, Germany).

Isolation and activation of neutrophils

The Ly-6G^{high}-expressing granulocyte subpopulation was isolated from bone marrow by positive selection magnetic cell separation (MACS) using the Miltenyi Biotec mouse Anti-Ly-6G MicroBead Kit according to the manufacturer's instructions (Miltenyi, Bergisch Gladbach, Germany). The purity of the isolated cells was at least 96% according to the expression of Gr-1 and CD11b.

Neutrophil receptor expression was determined by flow cytometry using fluorochrome-conjugated monoclonal antibodies.

2.5 × 10⁵ neutrophils were incubated with 100 ng ml⁻¹ LPS (*Escherichia coli* serotype 055:B5, Sigma-Aldrich) or with 2.5 × 10⁵ activated mast cells or mast cell-conditioned supernatants. After 5 h, the stimulation was stopped by washing cells with ice-cold FACS buffer (PBS + 0.5% BSA + 0.1% NaN₃). Then, cells were re-suspended in FACS buffer with added antibodies and incubated at 4°C for 15 min. The panel of monoclonal antibodies (eBioscience, Frankfurt, Germany) was PE-Cy7 anti-CD11b (clone M1/70), FITC anti-CD62L (clone MEL-14), APC anti-Gr-1 (clone RB6-8C5) and anti-CD16/CD32 (clone 93). Cells were washed twice with cold FACS buffer and were immediately analyzed using flow cytometry. Results are expressed as normalized geometric mean of fluorescence intensity on unstimulated neutrophils, which is a quantitative reflection of the number of CD11b or CD62L subset present on Ly-6G-positive cells.

ROS assessment

The amount of intracellular ROS was identified by flow cytometry using 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich).

When applied on the cells, the non-ionic, non-polar, colorless DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to non-fluorescent DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF) (41). Therefore, intracellular DCF fluorescence can be used as an index to quantify the overall ROS in cells.

2.5×10^5 neutrophils were incubated with 0.5 μM fMLP (Merck, Darmstadt, Germany) or with previously generated supernatant from 2.5×10^5 mast cells activated overnight. After defined time points, the experiment was stopped by washing cells with ice-cold FACS buffer (PBS + 0.5% BSA). Then, cells were re-suspended in FACS buffer with APC anti-Gr-1 (clone RB6-8C5), anti-CD16/CD32 (clone 93) and 5 μM DCFH-DA, and incubated at room temperature for 20 min avoiding direct light. Cells were washed twice with ice-cold FACS buffer and were immediately analyzed by flow cytometry. Results are expressed as normalized geometric mean of fluorescence intensity on unstimulated neutrophils.

In vitro phagocytosis assay

Neutrophil phagocytosis was quantified by ingestion of PC red fluorescent polystyrene microspheres (diameter 1 μm ; Fluoresbrite® polychromatic red microspheres; Polysciences, Eppelheim, Germany). Microspheres were opsonized with 20 $\mu\text{g ml}^{-1}$ IgG mouse antihuman antibody (clone 4C9) for 1 h at 37°C and subsequently were washed three times with PBS.

Aliquots of 0.25×10^6 freshly purified neutrophils were pre-incubated with supernatants from BMMC or with BMMC in co-culture in IMDM supplemented with 5% FCS (inactivated at 56°C) for 2 h at 37°C. Then, cells were incubated with microspheres (1 μl of suspension with 4.55×10^{10} particles per milliliter) for 30–45 min at 37°C. Cells were harvested and washed three times in cold FACS buffer. After staining with APC-conjugated anti-Gr-1 (clone RB6-8C5; BD) and PE-Cy7-labeled anti-CD11b (clone M1/70; eBioscience), cells were fixed with Cytfix (BD) and analyzed by FACS (Canto II; BD).

In vivo phagocytosis assay and bronchoalveolar lavage

Mice were anesthetized (Ketamin-ratiopharm®/Rompun 2%; Ratiopharm, Ulm, Germany) and challenged intra-nasally with 1 μg LPS in combination with 1.5×10^8 IgG-opsonized PC red fluorescent polystyrene microspheres. Eight hours later, animals were sacrificed and lungs were lavaged via the tracheal tube with PBS (1 ml). Numbers of bronchoalveolar lavage (BAL) cells were counted by trypan blue dye exclusion. Cells were then stained, fixed in 1% paraformaldehyde in PBS and analyzed by FACS.

Annexin V and propidium iodide apoptosis assays

Aliquots of 0.25×10^6 freshly purified neutrophils were incubated with supernatants from BMMC or with BMMC in co-culture for 20–24 h at 37°C. Cells were harvested and stained with APC-conjugated anti-Gr-1 (clone RB6-8C5; BD). After washing twice in PBS, cells were stained with FITC-labeled Annexin V according to the manufacturer's instructions (BD). Cells were stained with 50 $\mu\text{g ml}^{-1}$ propidium iodide (PI; Sigma Aldrich) and immediately analyzed by FACS.

Alternatively, DNA fragmentation was measured by flow-cytometric analysis of PI-stained nuclei (42).

Results

Mast cells modulate the expression of activation markers on neutrophils and trigger neutrophil oxidative burst

In order to investigate the influence of mast cells on neutrophil effector functions, we established a co-culture system using Ly-6G-positive MACS-purified neutrophils from murine bone marrow and *in-vitro*-generated BMMC. Prior to the onset of the experiments, BMMC were sensitized with monoclonal anti-DNP IgE. In co-culture with freshly isolated neutrophils, BMMC were activated upon cross-linking of IgE with the antigen, DNP-HSA. In pilot experiments, it was confirmed that neutrophils do not respond to either IgE-loaded BMMC or their supernatants or to the antigen, DNP-HSA (data not shown).

As depicted in Fig. 1, we first analyzed the expression of the activation markers CD11b and CD62L on the neutrophil surface using FACS analyses. Freshly isolated resting neutrophils and neutrophils activated by LPS in a range from 1 to 1000 ng ml^{-1} served as references. According to the expectations, resting neutrophils constitutively express intermediate levels of CD11b and high levels of CD62L on their surface, and the expression of both molecules is inversely regulated by LPS. Activation of neutrophils is accompanied by the rapid translocation of CD11b from latent intracellular granule-associated stores to the plasma membrane, whereas CD62L is down-regulated by shedding (43). Compared with untreated neutrophils, the expression levels of CD11b and CD62L are unimpaired in the presence of mast cells loaded with IgE but dramatically altered upon cross-linking of IgE with the respective antigen. Relative changes in the expression of both molecules following activation of mast cells exceed the effects of LPS on neutrophils, which was used as a positive control.

Modulation of CD11b and CD62L surface expression can also be achieved using culture supernatants from activated BMMC, implying that soluble mast cell mediators are mainly responsible for this phenomenon. Pro-inflammatory stimuli are known activators of neutrophil function and mast cell-derived TNF has already been shown to exert pleiotropic effects on different cell types and inflammatory responses (30). For this reason, we included mast cells derived from TNF- and GM-CSF-deficient mice in our studies. It can be concluded from the data shown in Fig. 1 that mast cell-derived TNF is partly, but not solely, responsible for this mast cell-mediated increase in CD11b expression, whereas TNF has hardly any effect on down-regulation of CD62L.

Conversely, up-regulation of CD11b on neutrophils is completely abrogated using conditioned medium derived from activated GM-CSF-deficient BMMC although lack of this cytokine does not impair the decrease of CD62L. It should be noted that all the effects of mast cell derived cytokines on neutrophils described herein were also verified using recombinant TNF and GM-CSF (data not shown).

Along the same line, we examined the ability of neutrophils to mediate an oxidative burst, a key event for the elimination of invading and ingested micro-organisms. Activation of the NADPH oxidase complex leads to the generation of ROS

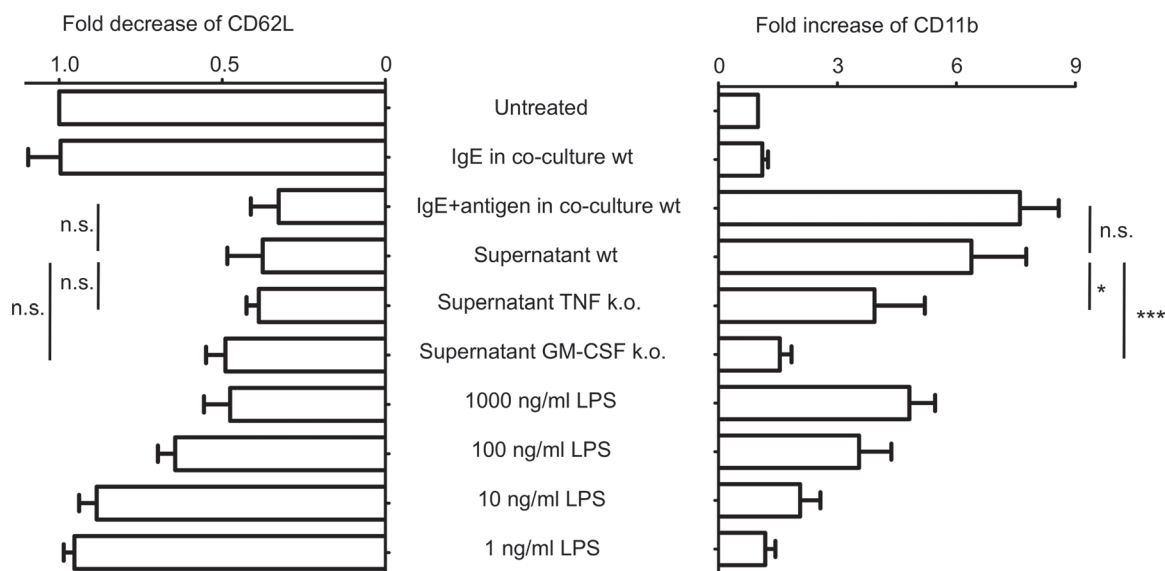


Fig. 1. Activated mast cells modulate the expression of activation markers on neutrophils. Freshly isolated neutrophils were incubated in co-culture in a ratio of 1:1 with IgE-loaded or IgE-loaded and cross-linked BMMC from wild-type mice. Additionally, neutrophils were treated with conditioned medium (50 vol%) derived from either activated wild-type BMMC (supernatant wt) or BMMC deficient for TNF or GM-CSF, respectively. Freshly isolated neutrophils and neutrophils treated with increasing doses of LPS served as references. After 5 h, CD62L and CD11b expression was measured by FACS as geometric mean fluorescence intensities. High expression of CD62L and constitutive low-level expression of CD11b on freshly isolated neutrophils were set to 1, respectively. Shown are the means (+ standard deviation) from at least three experiments. * indicates $P < 0.05$ and *** $P < 0.001$ as determined by one-way analysis of variance implemented in the Prism 5.0 software. n. s., not significant.

including H_2O_2 , which can be measured using the fluorescent dye DCF (44). On the basis of our observations shown above, we used conditioned medium from BMMC in order to investigate the impact of mast cells on the generation of ROS by neutrophils.

In Fig. 2A, incubation of neutrophils with supernatants derived from activated wild-type BMMC leads to a strong production of intracellular ROS seen as a rapid and transient increase in fluorescence intensities. As a positive control for these kind of experiments, we chose fMLP, a known activator of ROS generation, in concentrations ranging from 0.25 to 2 μM (Fig. 2B). However, the impact of mast cells on the generation of ROS equals that of fMLP at the highest concentrations (1 and 2 μM) and the oxidative burst initiated by mast cell-derived supernatants appears more sustained compared with fMLP. Furthermore, it can be seen that the oxidative burst mainly depends on mast cell-derived GM-CSF and although to a lesser extent on TNF.

Mast cells prolong the survival of neutrophils

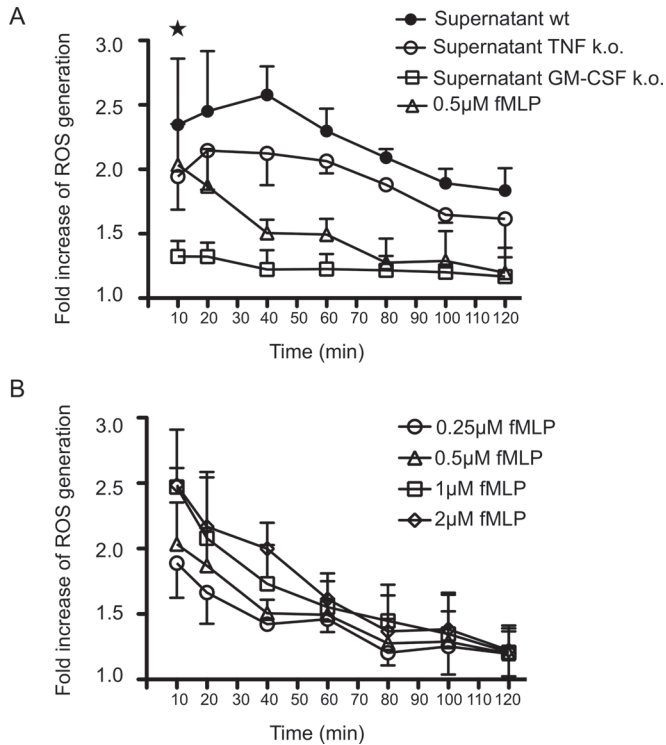
In order to investigate the impact of mast cells on spontaneous apoptosis of neutrophils, we incubated neutrophils in the presence of conditioned medium derived from activated BMMC (Fig. 3). Following 24 h incubation of neutrophils in medium, more than 40% of the cells bind annexin V, a sign for early apoptosis. A minor fraction of late apoptotic cells is in addition also positive for PI staining. The addition of supernatant derived from wild-type BMMC activated by cross-linking of IgE substantially decreases spontaneous apoptosis of

neutrophils (Fig. 3A and B). However, the antiapoptotic effect is completely abrogated using supernatants from GM-CSF-deficient BMMC (Fig. 3C and D). In contrast to this striking impact of mast cell-derived GM-CSF, TNF-deficient BMMC do not alter spontaneous neutrophil apoptosis (Fig. 3A and B). In these experiments, supernatants derived from IgE-loaded BMMC without cross-linking by antigen had no effect on neutrophil apoptosis (data not shown).

To support these findings, we also measured the DNA content in PI-stained neutrophil nuclei. As depicted in Fig. 3E, following 48-h incubation of neutrophils in medium, 50% of nuclei appear as a broad hypodiploid peak. The presence of conditioned medium derived from either activated wild-type or TNF-deficient mast cells strongly reduces the number of hypodiploid nuclei. Yet, supernatant from GM-CSF-deficient BMMC has hardly any effect on DNA fragmentation. Thus, both methods independently show that mast cell-derived GM-CSF can strongly delay the apoptosis of neutrophils.

Neutrophil phagocytosis is enhanced by mast cells

Ingestion and killing of microbes is a key element of neutrophil function. Thus, we next analyzed the ability of mast cells to promote neutrophil phagocytosis. This process can be quantitatively assessed by measuring the uptake of fluorescent polystyrene microspheres as depicted in Fig. 4. Without additional activation, about one-third of neutrophils take up beads, yet the relatively low fluorescence intensities indicate that most cells ingest only a very small number of beads (Fig. 4A). In the presence of LPS, both the numbers of cells that have taken up beads and their fluorescence



intensities strongly increase. However, phagocytosis can be further increased using supernatants derived from activated BMMC. Under these conditions, almost all neutrophils are loaded with numerous beads. In the absence of mast cell-derived TNF, the percentage of bead-positive neutrophils remains unchanged, yet the number of ingested particles per cell decreases (Fig. 4B). Finally, the lack of mast cell-derived GM-CSF leads to a strong decrease of both parameters. However, even in the absence of GM-CSF, the residual effect of mast cell-conditioned medium on neutrophil phagocytosis is still comparable with LPS. The enhancement of particle uptake is a very strong effect as displayed in Fig. 4C where conditioned medium derived from activated mast cells was diluted. Even at a dilution of 1:32, particle engulfment is significantly elevated above untreated levels. Additionally, we also varied the ratio of BMMC:neutrophils in co-culture experiments and got comparable results. At a ratio of 1:32, neutrophil particle uptake was still significantly increased (data not shown).

Thus, activated mast cells strongly boost neutrophil phagocytosis and it appears likely that, besides TNF and GM-CSF, other mast cell-derived mediators are able to promote this process.

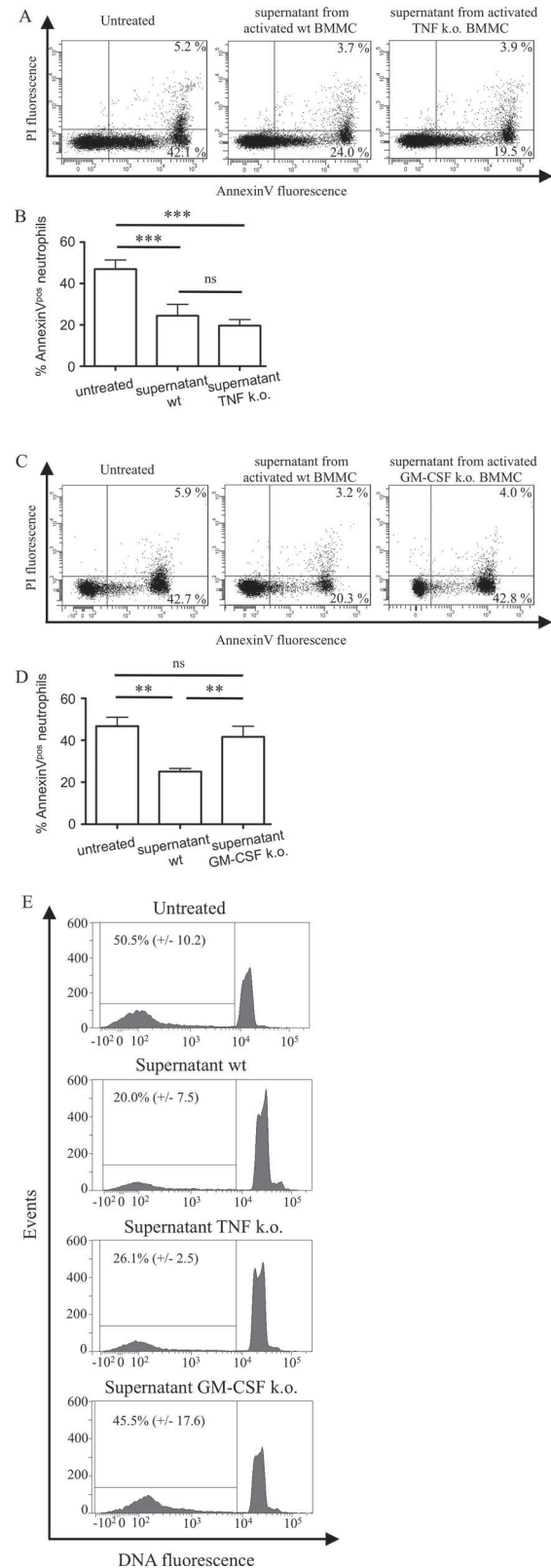


Fig. 3. Mast cells prolong the survival of neutrophils. (A) Freshly isolated neutrophils were incubated for 24 h with or without additional conditioned medium (50 vol%) derived from activated TNF-deficient BMMC or their congenic littermates. Spontaneous apoptosis was measured using staining with PI and annexin V. (B) Means (\pm standard deviation) from three experiments. * indicates $P < 0.05$ as determined by one-way analysis of variance implemented in the Prism 5.0 software. (C) Flow cytometry plots showing PI fluorescence vs Annexin V fluorescence for three conditions: Untreated (5.9% Annexin V+), Supernatant from activated wt BMMC (3.2% Annexin V+), and Supernatant from activated GM-CSF k.o. BMMC (4.0% Annexin V+). (D) Bar graph showing % Annexin V+ neutrophils for untreated, supernatant wt, and supernatant GM-CSF k.o. conditions. (E) Histograms showing DNA fluorescence for five conditions: Untreated (50.5% +/- 10.2%), Supernatant wt (20.0% +/- 7.5%), Supernatant TNF k.o. (26.1% +/- 2.5%), and Supernatant GM-CSF k.o. (45.5% +/- 17.6%).

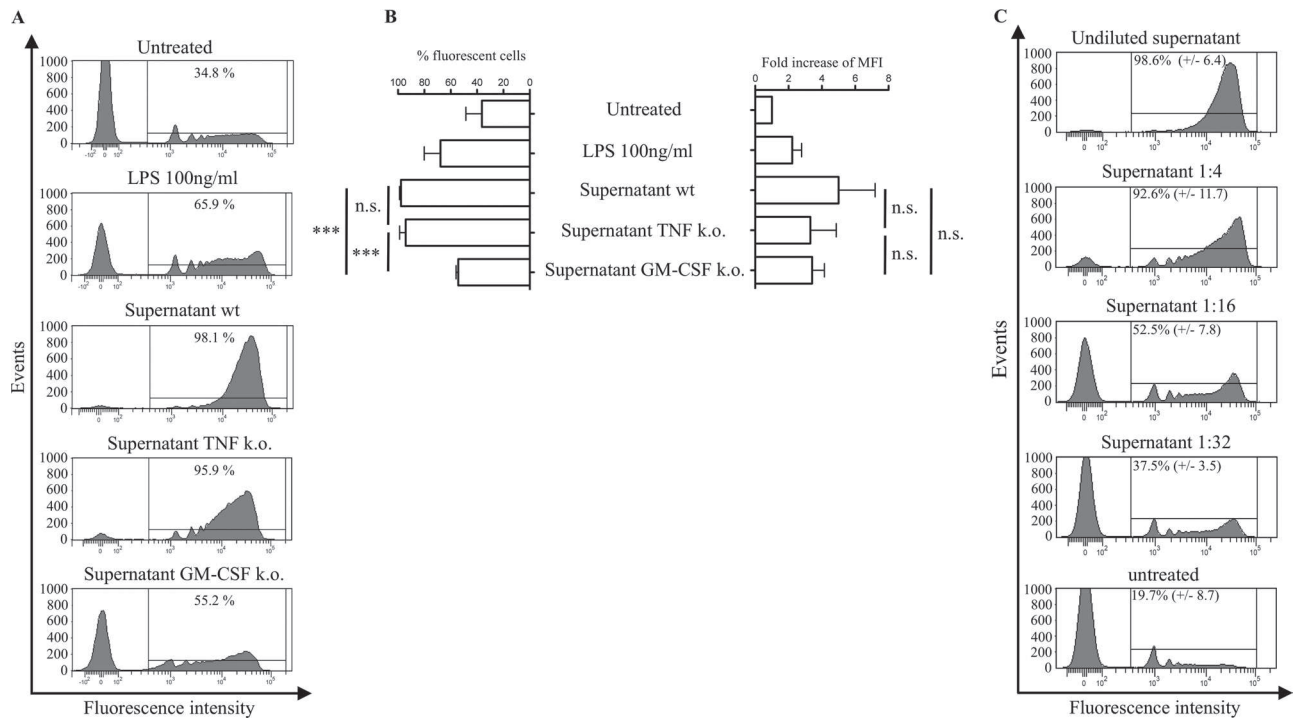


Fig. 4. Neutrophil phagocytosis is enhanced by mast cells. (A) Neutrophils were incubated for 2h with PE-labeled microspheres under the conditions shown, washed and FACS analyzed for the uptake of fluorescent particles. (B) Means (+ SD) of three experiments like the representative shown in (A). (C) Neutrophils were treated as described above but only conditioned medium derived from activated wild-type mast cells was diluted as indicated. Means (\pm standard deviation) from three experiments. *** $P < 0.001$; n. s., not significant.

Mast cells boost neutrophil phagocytosis in a model for acute lung inflammation

In order to investigate whether mast cells also boost the function of neutrophils *in vivo*, we chose a model of LPS-induced acute lung inflammation. Besides wild-type and mast cell-deficient *Kit^{W-sh}/Kit^{W-sh}* mice, mast cell-deficient mice were also engrafted with BMMC derived from wild-type, TNF- and GM-CSF-deficient mice 12 weeks prior to the experiment. Successful engraftment of *Kit^{W-sh}/Kit^{W-sh}* mice was ensured by staining lung sections derived from all animal groups for the presence of mast cells (Fig. 5, lower panel).

For the induction of pulmonary inflammation, LPS was given intra-nasally in combination with fluorescent polystyrene microspheres. Eight hours later, cellular infiltrates were recovered by lavage of the lungs and analyzed for the presence of neutrophils. As depicted in Fig. 5 (upper panel), application of LPS induces a strong influx of neutrophils into the lungs, which is comparable in all experimental groups. This mast cell-independent recruitment of neutrophils is most likely due to the fact that in the lungs different cell types, including structural cells, express TLR4 and are able

to initiate an innate immune response (45). Phagocytosis of fluorescent beads by neutrophils can be visualized by FACS (Fig. 5, middle panel). Most importantly, the absence of mast cells is accompanied by a reduction in neutrophil phagocytosis, measured as a drop in fluorescence intensities. This reduction in neutrophil phagocytosis can be corrected through the selective repair of mast cell-deficiency with mast cells derived from wild-type or TNF-deficient mice. Obviously, the observed effect of mast cell-derived TNF on neutrophil phagocytosis *in vitro* (Fig. 4) can be compensated for by additional sources for TNF in the lungs, most likely bronchiolar epithelial cells and alveolar macrophages (46). This assumption is supported by our observation that TNF levels in BAL do not significantly differ between mast cell-deficient and wild-type mice at 8h after application of LPS (data not shown).

However, engraftment of sash mice with GM-CSF-deficient BMMC is insufficient to restore neutrophil phagocytosis, indicating that mast cells serve as a non-redundant source for this cytokine in this model. Unfortunately, we were not able to measure GM-CSF in BAL using ELISA. In summary, this result corroborates our data generated *in vitro* and emphasizes the influence of mast cells on neutrophil phagocytosis *in vivo*.

Discussion

It is increasingly being recognized that mast cells are able to induce and to regulate inflammatory responses in diverse experimental settings. These cells are strategically located

deviation) from four experiments like the one shown before. (C) Performed as described above but to investigate the effect of GM-CSF-deficient BMMC on neutrophil apoptosis. (D) Means (+ standard deviation) of three experiments. (E) Neutrophils were treated as described in (A) but incubated for 48h. Following PI staining, apoptotic nuclei appear as a broad smear of low fluorescence intensity. Means (\pm SD) from three experiments are given. ** $P < 0.01$; *** $P < 0.001$; n. s., not significant.

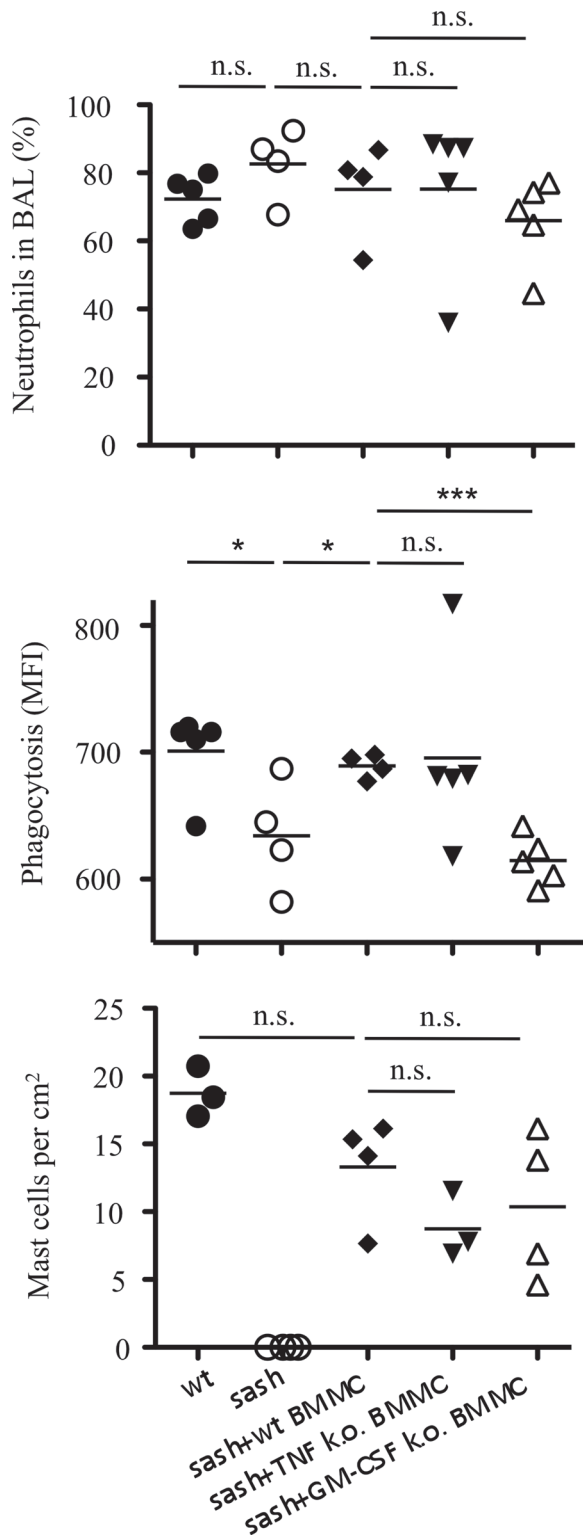


Fig. 5. Mast cells promote neutrophil phagocytosis in a model for acute lung inflammation. One microgram LPS in combination with 1.5×10^8 fluorescent polystyrene microspheres were applied intranasally in anesthetized mice of the indicated groups. Eight hours later, BAL was recovered and neutrophils were counted (upper panel). FACS analyses were performed to quantify neutrophils that have undergone phagocytosis of PE-labeled microspheres. Geometric mean fluorescence intensities (MFI) are shown. * indicates $P < 0.05$

at the borders between host and environment, that is skin and mucosal surfaces, and can rapidly respond to a variety of stimuli even in the absence of IgE antibodies (30, 47). Activation of mast cells can promote the rapid recruitment of neutrophils that are able to combat harmful intruders using oxidative and non-oxidative mechanisms but these processes can also play a key role in the development of inflammatory and autoimmune diseases (48). Further, in the context of intercellular communication, recent studies revealed a bidirectional cross-talk between mast cells and eosinophils, termed the allergic effector unit, augmenting the activation of both cells (49, 50).

Our observations reported herein demonstrate that mast cells are able to boost important effector functions of neutrophils, that is phagocytosis and generation of ROS. This is partly due to the *de novo* production of TNF and GM-CSF, whereas histamine, which is rapidly released from granular stores, had no effect on neutrophils (data not shown). It was reported that mast cells are able to store TNF that can be released rapidly upon stimulation, but it should be noted that BMMC used for the experiments described herein and other mast cell lines do not contain preformed TNF (20). It has been known for a long time that mast cells are able to produce GM-CSF (51) and our results show prominent effects of mast cell-derived GM-CSF on all parameters of neutrophil activity examined including its strong antiapoptotic action. A variety of inflammatory signals can extend the lifespan of neutrophils. TNF is also known to regulate neutrophil apoptosis but opposing effects have been reported in that it can either accelerate or delay programmed cell death, depending on the concentration. High concentrations of TNF promote apoptosis, whereas low concentrations are antiapoptotic. This dual effect of TNF is likely due to differential effects on the antiapoptotic pathways in neutrophils, that is accelerated turnover of the antiapoptotic protein Mcl-1 at high concentrations and increased expression of another antiapoptotic molecule, Bcl-1, at low concentrations of this cytokine (52).

However, in our *in vitro* system, mast cell-derived GM-CSF is solely responsible for delaying apoptosis of neutrophils. This antiapoptotic effect of mast cells might support efficient anti-microbial activity in inflamed tissues.

Besides mast cell-derived cytokines, we have good evidence that mast cell-derived lipid mediators also strongly promote the activation of neutrophils (data not shown) but additional work is needed to identify these lipids.

To corroborate our *in vitro* findings, we used a model for acute lung inflammation following instillation of LPS. Inhalation of LPS leads to the rapid recruitment of neutrophils and also activates mast cells (53, 54). In agreement with another report (16), we found that LPS-induced airway neutrophilia is not influenced by the lack of mast cells but importantly, the ability of neutrophils to phagocytose is impaired in the absence of mast cells.

and *** $P < 0.001$ as determined by unpaired *t*-test implemented in the Prism 5.0 software (middle panel). Lung sections were stained for the presence of mast cells using toluidine blue (lower panel). Representative of at least two experiments with 4–5 mice per group.

Taken together, our results suggest that mast cells are involved in the local control of neutrophil activation and survival and thus could have important implications for the role of mast cells in innate immunity but also in allergic and autoimmune diseases.

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